Obstructive sleep apnoea syndrome and HLA in the North of Portugal

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Introduction. The obstructive sleep apnoea syndrome (OSAS) is a common, complex and polygenic disease with diverse aetiologies interacting to produce a single phenotype. OSAS occurs throughout the entire lifespan and familial aggregation has been suggested. Several predisposing factors, as age, gender and obesity have been described. Associations between HLA polymorphisms and sleep disorders are confirmed, in European and Non-European descendent populations. However the associations found between HLA alleles and OSAS have not been consistent and have no informative value for sleep disorder classification.

Aims. To explore the genetic association of HLA with OSAS in a northern Portuguese population and to evaluate the role of obesity in the context of HLA in OSAS.

Patients and methods. A cohort of 131 patients with OSAS was studied. Patients followed up in an Outpatient Sleep Clinic were assessed by clinical history, night sleep polygraphic recording, multiple sleep latency test (when necessary for differential diagnosis), laboratorial and demographic studies. A control population (CP) of 223 healthy individuals was used for comparison. HLA-DRB1 genotyping was performed using a polymerase chain reaction with sequence specific primers methodology.

Results. In this cohort, the HLA-DRB1*03 allele was identified as a susceptibility factor for OSAS (24% OSAS vs. 15% CP; p = 0.025; odds ratio = 1.861; 95% CI = 1.081-3.205). No significant differences were found for other HLA-DBR1* alleles.

Conclusion. HLA-DRB1*03 is a susceptibility factor for OSAS in Portuguese population.

Key words. Gender. HLA-DRB1*03. Obesity. Obstructive sleep apnoea syndrome (OSAS). Risk factors. Sleep disorders.

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Introduction

Obstructive sleep apnoea syndrome (OSAS) is a common but complex and polygenic disease with various predisposing factors interacting to produce a single phenotype [1-4]. The clinical features are the repetitive episodes of total (apnoea) or partial (hypopnoea) upper airway obstruction that occur during sleep even though respiratory effort is still present [5,6]. The severity of OSAS is quantified by an Apnoea-Hypopnoea Index (AHI), referring to the number of apnoea or hypopnoea episodes per hour. It is considered relevant if five or more episodes occur. Patients frequently present loud and habitual snoring, witnessed apnoea, un-refreshing sleep and excessive daytime sleepiness [5].

OSAS occurs throughout the entire lifespan [1] and in western countries affects approximately 2-4% of males and 1-2% of females in the middle-aged population (40-60 years-old) [2,5,7]. In post-menopausal women (especially in those without hormone replacement) this prevalence increases,

although it remains lower than in men in the same age range [2,8]. The American Academy of Sleep Medicine, point values of 3-7% of adult men and 2-5% of women suffering of OSAS [5]. The disorder is frequently unrecognized and undiagnosed, because patients interpret their symptoms as a normal manifestation of poor lifestyle.

Several predisposing factors namely obesity, but also age and gender, have been implicated in this syndrome. Anatomical alterations of bone or soft tissues from craniofacial structures (nasal, mouth, or oropharynx) and other conditions associated with nasal obstruction frequently contribute to OSAS. Upper airway calibre restrictions are a result of multiple factors [5,6]. Such diverse factors influence the AHI, oxy-haemoglobin desaturation and hypercapnia, common consequences of this complex disorder [1,5,7,9].

Obesity and overweight do influence risk and OSAS severity. According to the World Health Organization obesity is a common ('endemic') disease in developed countries [10] and represents a risk to

health due to the associated morbidity and mortality [10,11]. Obesity and overweight are referred to the Body Mass Index (BMI). BMI is calculated as a function = Weight (kg) / [Height (m)]^2. The cut-off for an overweight person is a BMI between 25.0 and 29.9 and for an obese person is BMI ≥ 30 [10,11]. Obesity increases the risk of OSAS [3]. In contrast, weight loss may reduce the severity of this condition [3,12].

In terms of identifying causal genes to OSAS, several genome linkage analyses and candidate gene association studies have been performed [13, 14]. The heterogeneity of clinical OSAS phenotypes difficult the identification of causal genes[4]. Familial aggregation [9] and inherited factors account for approximately 40% of the risk of OSAS [1,3], but is unlikely that a single genetic factor exists. As obesity is a strong predisposing factor for OSAS the analysis of genetic markers could be biased. Therefore genetic studies on OSAS should distinguish between patients with and without obesity.

One of the most reported genetic association studies in sleep disorders has been with the major histocompatibility complex (MHC), in humans known as human leukocyte antigen (HLA). The HLA is a genetic marker that may be implicated in the pathogenesis of a disease *per se* or through a gene located in the same region (MHC) that may contribute to disease susceptibility/protection. The first description of the association between HLA polymorphisms and sleep disorders was reported in Japanese patients. The association between narcolepsy and HLA-DR2 was presented in 1983 [15] and the results published by the same group in 1984 [16]. Later, it was discovered that the HLA-DQB1*06:02 allele, rather than the HLA-DR2 antigen is a better marker for narcolepsy across all ethnic groups [17,18]. In some populations, 90-100% of patients with type 1 narcolepsy carry this allele, often in combination with HLA-DRB1*15:01 (HLA-DR2), which are in full linkage disequilibrium [19]. Studies of microsatellites in the HLA-DQ region indicate that the susceptibility factor is located in this region [20]. Individuals homozygous for the HLA-DQB1*06:02 allele have an increased risk (2-3 times) to develop narcolepsy. Heterozygotes for DQB1*06:02 in combination with other HLA class II alleles (including DQB1*03:01, DQA1*06, DRB1*04, DRB1*08, DRB1*11 and DRB1*12) are also at risk. It has also been recognized that several HLA-DQB1* alleles (non-HLA-DQB1*06:02) act as protective factors [21,22].

However the association of HLA alleles/antigens with OSAS is much less clear [23]. In 2005, Brunetti et al reported in a southern Italy population [24],

that HLA-A*33, DRB1*03 and DQB1*02 were significantly more frequent in patients with OSAS than in healthy individuals. However, this significance was lost after Bonferroni's multiple testing corrections [24]. A study carried out in Korean patients [25] revealed an association between OSAS and HLA-A*11 and DRB1*09 genes. The HLA-A*11 allele frequency was significantly lower in patients with OSAS (acting as a protective factor) and the HLA-DRB1*09 allele frequency was higher when compared with controls (acting as a susceptible factor). They also stressed that HLA-DRB1*08 allele is associated with disease severity [25]. More recent studies show changes on the ratio of electroencephalographic sleep waves frequencies in HLA-DQB1*06:02 positive OSAS patients, opening the discussion about the role of HLA alleles in the sleep structure [26].

The present study aims to contribute to the discussion of the reported differences on HLA frequencies found in OSAS patients by analysing such frequencies in an unselected and well-controlled OSAS population from the North of Portugal. We also investigate if such frequencies were independent from possible confounders represented by the two most common OSAS risk factors: obesity and gender.

Patients and methods

Study population

A random cohort of 371 patients (253 males and 118 females) complaining of diverse sleep disturbances, assessed consecutively at the Sleep Outpatient Clinic of Hospital Santo António/CHP–Porto (a tertiary hospital in the North of Portugal) was enrolled in this study.

Patients were evaluated by means of clinical history, physical and neurological evaluation, night sleep polygraphic EEG-video recording, that included EEG, EOG, EMG –chin and lower limbs–, nasal and mouth ventilation and thoracic respiratory effort, O_2 saturation, EKG, and snoring. Additionally, for patients with suspicion of narcolepsy type 1 or type 2 and for hypersomnia, subsequent multiple sleep latency test (MSLT) was performed the day after night registration. A blood venous sample, for laboratory studies, was collected in the same day of sleep night registration. The study was approved by the Hospital Ethical Committee.

The medical records were reviewed by the participating clinicians and confirmed by the study coordinator. Patients were then classified according to

Table I. Demographic characteristics of obstructive sleep apnoea syndrome patients.

	All patients			Male			Female		
	n	%	Mean	n	%	Mean	n	%	Mean
Number of patients	131	–	–	114	87.0	–	17	13.0	–
Age (years)	–	–	55.1	–	–	55.0	–	–	55.5
Patients with age < 50 years	38	29.0	40.9	34	29.8	40.6	4	23.5	43.2
Patients with age ≥ 50 years	93	71.0	60.9	80	70.2	61.2	13	76.5	59.2
BMI (kg/m²)	127 [a]	–	29.4	110	–	29.4	17	–	29.9
Patients with BMI < 30	77	60.6	26.4	68	61.8	26.6	9	52.9	24.7
Patients with BMI ≥ 30	50	39.4	34.2	42	38.2	33.9	8	47.1	35.7

BMI: Body Mass Index. [a] Information is not available for four patients.

the International Classification of Sleep Disorders –Third Edition (ICSD3) criteria [5]. From the initial cohort, 131 subjects (35.3%) exhibited OSAS (AHI > 5) –114 males and 17 females. The remaining cohort (n = 240; 139 males and 101 females) had other sleep disorders and were considered as non-OSAS patients and include: 58 patients (41 males and 17 females) with primary snoring or snoring associated to respiratory distress with an AHI < 5; and 182 patients with other sleep disorders mainly insomnia, periodic limb movement disorders, narcolepsy, idiopathic hypersomnia or other hypersomnias. All but four OSAS patients and 142 of non-OSAS had BMI calculated. The control group (CP) comprised 223 healthy individuals clinically assessed, ethnically matched and recruited among northern Portuguese blood donors.

DNA extraction and genotyping

Peripheral blood venous samples (10 mL) from sleep patients and controls were collected in EDTA (ethylenediaminetetraacetic acid). Each subject was informed about the study and gave written informed consent for the genetic analysis.

The genomic DNA was obtained from proteinase-K treated peripheral blood leukocytes by a salting-out procedure [27]. DNA was amplified by polymerase chain reaction with sequence-specific primers (PCR-SSP) for the identification of HLA-DRB1 genes, based on methods and primer sequences previously described [28]. PCR products were visualized under ultraviolet light after electro-phoretic separation on 1.5% agarose gel containing ethidium bromide. Genotypes were deduced from the amplification patterns.

Statistical analysis

HLA-DRB1* phenotype frequencies were determined by direct counting. HLA frequencies in OSAS and non-OSAS patients and controls were compared using the Pearson chi-square test or the Fisher's exact test, as appropriate. Odds ratios (OR) and their respective 95% confidence interval (95% CI) were calculated. Values of $p < 0.05$ were considered as statistically significant.

To identify further the specific alleles contributing to OSAS susceptibility, a stepwise logistic regression, on an allelic level using backward selection, was applied. Starting from a model with all HLA-DRB1 alleles, the least significant allele was removed one at the time until all remaining alleles were significant, based on the likelihood-ratio test. It should be noted that ORs obtained in a multivariate logistic regression analysis were adjusted for all the other alleles included in the model, and differ from those obtained when a given allele is compared with all other alleles. Data were analyzed using SPSS v. 21 software.

Results

The demographic characteristics of the 131 OSAS patients are summarized in table I.

Table II. HLA-DRB1* allele frequencies in Portuguese patients with obstructive sleep apnoea syndrome (OSAS) and in the control population.

	Controls (n = 223)	OSAS (n = 131) [a]	p	OSAS/obese (n = 50)	OSAS/non-obese (n = 77)	p
DRB1*01	48 (22%)	28 (21%)	0.973	15 (30%)	12 (16%)	0.075
DRB1*03	33 (15%)	32 (24%)	0.024	15 (30%)	16 (21%)	0.292
DRB1*04	52 (23%)	28 (21%)	0.673	10 (20%)	18 (23%)	0.827
DRB1*07	58 (26%)	38 (29%)	0.540	13 (26%)	25 (33%)	0.552
DRB1*08	19 (9%)	16 (12%)	0.261	4 (8%)	12 (16%)	0.278
DRB1*09	13 (6%)	2 (2%)	0.052	0	2 (3%)	0.519
DRB1*10	7 (3%)	3 (2%)	0.642	0	3 (4%)	0.278
DRB1*11	44 (20%)	30 (23%)	0.479	10 (20%)	19 (25%)	0.666
DRB1*12	8 (4%)	4 (3%)	0.789	2 (4%)	2 (3%)	0.646
DRB1*13	68 (30%)	31 (24%)	0.167	14 (28%)	16 (21%)	0.396
DRB1*14	14 (6%)	8 (6%)	0.949	4 (8%)	3 (4%)	0.432
DRB1*15	47 (21%)	21 (16%)	0.245	7 (14%)	14 (18%)	0.629
DRB1*16	9 (4%)	8 (6%)	0.379	3 (6%)	5 (7%)	1.000

[a] Information is not available for four patients.

The distribution by gender shows that, in the OSAS cohort, the frequency of males was higher (87% males vs. 13% females). The mean age of patients was 55.1 years old and similar values were found (55 years old in males and 55.5 years old in females) in both genders. As the age peak incidence is about 50 years old we calculated the distribution of OSAS patients under and over 50 years old that is similar in both genders (males: 29.8% < 50 years old, 70.2% ≥ 50 years old; females: 23.5% < 50 years old, 76.5% ≥ 50 years old) (Table I).

To investigate the effect of HLA genetic determinants on susceptibility to OSAS, patients and controls were genotyped for HLA-DRB1*alleles (Table II). The HLA-DRB1*03 allele frequency was higher in the OSAS population when compared with controls (24% OSAS vs. 15% CP). This difference is statistically significant ($p = 0.024$; OR = 1.86; 95% CI = 1.08-3.21). For the others HLA-DRB1 alleles tested, no association was found. To verify the independence of the HLA-DRB1*03 allele relative to the presence of the other HLA alleles tested, a logistic regression analysis was carried out. The re-sult obtained confirm the association of DRB1*03 allele with OSAS ($p = 0.025$; OR = 1.861; 95% CI = 1.081-3.205). When OSAS patients were sub grouped in obese and non-obese, no differences in HLA allele frequencies were found between the two sub groups (Table II).

In order to understand if obesity increases the risk of OSAS [5,7,27], the patient cohort was divided in two sub-groups according to BMI status (< 30 or ≥ 30) and the frequency of obesity was compared with the data published for the Portuguese population [11]. From the 127 OSAS patients in which the BMI was calculated, 39.4% were obese, and the remaining 60.6% non-obese. The percentage of obese subjects was significantly higher in the group of OSAS patients than the one reported for the general Portuguese population (39.4% vs. 14.2%; $p <$ 0.0001; OR = 3.93; 95% CI = 2.74-5.63). Additionally the frequency of obesity was assessed in the sub-group of 142 non-OSAS randomly selected from the initial cohort of 371 patients, in which BMI was available. In this group, 42 patients (29.6%) were obese (BMI > 30) and 100 were not. This difference

was not statistically significant (39.4% vs. 29.6%; $p = 0.096$). To note that the percentage of obese individuals in the non OSAS subgroup was also significantly higher than the one reported for the general Portuguese population (29.6% vs. 14.2%; $p < 0.0001$; OR = 2.54; 95% CI = 1.76-3.66).

The mean value of BMI was 29.4 in males and 29.9 in females. Non-obese OSAS patients had a BMI of 26.6 in males and 24.7 in females and obese OSAS patients had a BMI 33.9 in males and 35.7 in females. To access if the presence of the DRB1*03 allele influences obesity, OSAS patients were divided in non-obese (BMI < 30) and obese (BMI ≥ 30). The frequency of this allele was lower in non-obese than in obese patients (20.8% vs. 30%, respectively), but the difference observed was not statistically significant (Table III).

Additionally we addressed the same question in the non-OSAS group. Also no statistically significant differences were observed (9.5% vs. 16%, respectively) between obese and non obese patients.

Discussion

As already stated, the main goal of the present study was to investigate the association between HLA and OSAS in patients with sleep disorders of the North of Portugal.

The demographic characteristics of Portuguese OSAS patients reproduce those of other populations [5,8] with a higher frequency of male subjects (87% males vs. 13% females; gender ratio 7:1) confirming that male gender is a general risk factor for OSAS (male/female ratio 4:1 to 10:1) [8].

It was also found that OSAS is most common in middle-aged patients, with a mean age of 55.1 years (range: 6-84 years). OSAS is known to occur in a wide age range, increasing with age, with a plateau in the elderly [5].

A significant association between the HLA-DRB1*03 allele and OSAS was demonstrated in this study (24% in OSAS vs. 15% in CP; $p = 0.025$). This association is independent of gender. These results concur with those observed by Brunetti et al in which the HLA-DRB1*03 allele is overrepresented in Italian children with OSAS (24.4% vs. 12.8%; $p = 0.03$) [24]. At present there is no answer to the question of how HLA do influence OSAS. Both results, the present study in adults and those in children [24] address such question and stimulate some discussion. As a comment for such discussion we note that OSAS in children and adults may have different pathophysiological basis. However this is

Table III. HLA-DRB1*03 prevalence in obstructive sleep apnoea syndrome patients (obese/non obese) ($n = 127$) [a].

		HLA-DRB1*03
BMI < 30	All ($n = 77$)	16 (20.8%)
	Male ($n = 68$)	13 (19.1%)
	Female ($n = 9$)	3 (33.3%)
BMI ≥ 30	All ($n = 50$)	15 (30.0%)
	Male ($n = 42$)	13 (30.9%)
	Female ($n = 8$)	2 (25.0%)

BMI: Body Mass Index. [a] Information is not available for four patients.

not true for all OSAS patients. The HLA-OSAS association found in these populations may indicate that certain individuals exhibiting such genetic characteristics are more prone to have sleep respiratory distress (OSAS). Other way to comment is based on the hypothesis stated by Riha et al, considering that 'the genotype of obstructive sleep apnoea/hypopnoea syndrome is affected by the lack of a consistent definition of the phenotype [...] at a single point in time' [1]. The concept, of individualization of a significant result at a 'single point in time' in OSAS patients may also help to understand the results obtained in different ethnic or demographic populations.

In fact, studies conducted in Asian and other European OSAS populations have shown different HLA associations. A Japanese study [29] with a cohort of 32 male subjects with OSAS, failed to demonstrate an association between HLA-DR alleles and OSAS patients. Also a Korean study [25], with 25 patients (24 males, 1 female) with OSAS, reported that HLA-DRB1*09 allele frequency was increased (23% OSAS vs. 16% CP). On the same study, the authors restricted the analysis to the most severe apnoea cases and reported an increase of the HLA-DRB1*08 allele frequency (36% in OSAS vs. 20% in CP). Our study did not support these findings. Both studies are relevant, but altogether analysed scarcely representative cohorts. It should be noted that other factors such as ethnic backgrounds or inherent demographic characteristics, the most noticeable being the gender ratio, could be implicated.

The Japanese study previously referred [29] shows that 'HLA-A2 positive patients were more obese than HLA-A2-negative patients' and 'that the high-

er incidence of OSAS in patients was due to the influence of this genetic marker on obesity' [29]. The results of this study were considered later as biased by the presence of a significantly higher number of obese individuals among the patients with OSAS when compared to controls [24]. As for other diseases the interactions between HLA genotype and BMI status are still under discussion supporting a role for HLA in obesity [30].

The prevalence of obesity (39.4%) in our cohort is almost triple than the one reported for the Portuguese population (14.2%) [11]. The obtained results show that obesity increases the risk of developing OSAS three to four times (OR = 3.93) in agreement with studies documenting that obesity is a common and pathogenic factor for OSAS in adults [3,5,12,31-33].

In the present study we found that OSAS is associated with HLA-DRB1*03 allele and is also associated with obesity. In order to study the possible confounder effect of obesity on OSAS alleles frequencies, we conducted a regression analysis adjusting for age, sex and BMI to clarify and avoid possible bias of the influence of this allele in obesity. The results of this analysis reinforce our conclusion that HLA-DRB1*03 is associated with OSAS and that such association is independent of obesity in this group of patients.

In conclusion, the relationship between OSAS, HLA alleles and risk factors (obesity and gender) was studied for the first time in a Portuguese population, and to the best of our knowledge is the larger cohort analysed so far. We found that HLA-DRB1*03 allele is a susceptibility factor for OSAS, and that being male and obese increases the risk for OSAS. These results need to be confirmed in other populations and in larger cohorts.

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Síndrome de apnea obstructiva del sueño y HLA en el norte de Portugal

Introducción. El síndrome de apnea obstructiva del sueño (SAOS) es una enfermedad frecuente, compleja y poligénica, con diversas etiologías que interaccionan originando un fenotipo único. El SAOS puede ocurrir a cualquier edad del individuo y se presume la existencia de agregación familiar. Han sido descritos diversos factores de predisposición, como la edad, el sexo y la obesidad. La relación entre los polimorfismos del antígeno leucocitario humano (HLA) y trastornos del sueño está confirmada, tanto en poblaciones europeas como no europeas. No obstante, las relaciones descritas entre los alelos HLA y SAOS no han sido coherentes y carecen de valor informativo para la clasificación del trastorno del sueño.

Objetivo. Explorar la asociación genética del HLA con el SAOS en una población del norte de Portugal y evaluar el papel de la obesidad en el contexto del HLA en el SAOS.

Pacientes y métodos. Se estudió una cohorte de 131 pacientes con SAOS. Los pacientes fueron atendidos en una clínica del sueño ambulatoria donde se valoraron los antecedentes clínicos, se les practicó una polisomnografía nocturna, una prueba de latencia múltiple del sueño (si lo exigió el diagnóstico diferencial), analíticas y estudios demográficos. A efectos comparativos, se utilizó una población de control de 223 personas sanas. Se efectuó el genotipado del HLA-DRB1 con la reacción en cadena de la polimerasa mediante cebadores de secuencia específica.

Resultados. En esta cohorte, el alelo HLA-DRB1*03 fue identificado como un factor de predisposición para el SAOS (24% del SAOS frente a 15% de la población de control; *p* = 0,025; *odds ratio* = 1,861; intervalo de confianza al 95% = 1,081-3,205). No hubo diferencias significativas en lo referente a otros alelos HLA-DBR1*.

Conclusión. El HLA-DRB1*03 es un factor de predisposición para el SAOS en la población portuguesa.

Palabras clave. Factores de riesgo. HLA-DRB1*03. Obesidad. Sexo. Síndrome de apnea obstructiva del sueño (SAOS). Trastornos del sueño.